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Scanning Electron Microscopic Study of Virulent *Yersinia pestis* and *Yersinia pseudotuberculosis* Type I

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Virulent *Yersinia pestis* was grown on heart infusion blood agar and examined by scanning electron microscopy, exposing the fraction 1 envelope antigen on cell surfaces as a lumpy coating that spilled into the surrounding milieu. The amount of antigen depended on both the duration and temperature of incubation. Comparison of the cultures grown at 37°C for 24, 48, and 72 h with those grown at 22°C for identical periods demonstrated that the antigen increased in amount with the length of incubation, and that the overall production of antigen was much greater at 37°C than at 22°C. These experiments visually confirmed the findings of our previous immunological studies. Moreover, we established that the closely related, virulent organism *Y. pseudotuberculosis* bears no such antigenic coating at any temperature or incubation period. In addition, the emergence of multiple flagella was noted when *Y. pseudotuberculosis* was grown at 22°C in a liquid medium, whereas *Y. pestis* remained without these organelles. These observations perceptibly corroborated the absence of fraction 1 envelope antigen and the presence of flagella, respectively, for distinguishing *Y. pseudotuberculosis* from *Y. pestis*.

Earlier studies (2, 4, 12) reported that the fraction 1 envelope antigen of *Yersinia pestis* is of paramount importance in immunizing against plague. As long ago as 1914, Rowland (14) originally demonstrated that the envelope antigen could be enhanced simply by raising the incubation temperature to 36°C. In 1932, Schütze (15) again proved that the antigen could be produced in quantity only by fully virulent cultures grown at 37°C.

Some 20 years later, Amies (1) found that in cultures grown at 37°C, such an "envelope" could be demonstrated by means of a wet India ink preparation illuminated by a dark-field condenser. The envelope emerges as a dark area surrounding the bacterial cells, and when the bacteria are grown under favorable conditions, the width of the envelope may exceed that of the cell itself. Subsequently Chen et al. (7) grew virulent *Y. pestis* on nutrient agar at diverse temperatures for 72 h, estimating yields of fraction 1 envelope antigen by the reverse complement fixation test. The highest yield came from pathogens grown at 37°C. A few years later, Crocker and his co-workers (9), using virulent *Y. pestis* grown on a collodion film over nutrient agar at 37°C for 24 h, produced electron micrographs illustrating sharply outlined, highly electron-dense bodies embedded in a material of low electron density—the envelope antigen—which was homogeneous throughout and easily washed from the film with distilled water. More recently, we studied the development and ultrastructure of fraction 1 envelope antigen in both virulent and avirulent strains of *Y. pestis* growing for various periods at a temperature of 37°C (5). We found that the maximal amount of extracellular envelope material could be obtained when the cells were incubated at 37°C for 72 h.

The intent of the present experiments was to substantiate our previous fine-structural evidence and to visualize the antigen on cell surfaces by means of scanning electron microscopy (SEM). Concomitantly, we investigated the evolution of flagella on the antigenically related, virulent *Y. pseudotuberculosis*.

MATERIALS AND METHODS

Microorganisms. The virulent species *Y. pestis* T1 (5) and *Y. pseudotuberculosis* (type I) were maintained in a lyophilized state at 4°C, with transferal of selected working cultures to heart infusion blood agar slants.

Procedures. For SEM studies, the *Y. pestis* was grown on two heart infusion blood agar slants, one incubated at 22°C and the other at 37°C. Cultures were fixed in situ with 2% glutaraldehyde in Dulbecco modified Eagle medium for 1 h after 24, 48, and 72 h of growth at each temperature. They were then transferred to polylysine-coated cover slips and

fixed for an additional 15 min. After several rinses with distilled water, the cultures were dehydrated in a graded series of ethanols, followed by a second series of ethanol Freon 113, and critical-point-dried with Freon 13 (8). They were then coated with 20.0-nm layer of gold-palladium and subsequently examined and photographed in a Cwickscan field-emission SEM.

The highly purified, soluble fraction 1 envelope antigen of *Y. pestis* (1 mg/ml in saline) was originally prepared for plague immunization and used as the specific fraction 1 antigen for complement fixation and hemagglutination antibody tests (2, 6). The antigen was fixed by the addition of an equivalent volume of 4% glutaraldehyde in neutral 0.1 N $NaPO_4$ buffer. After 1 h of fixation, the solution was transferred to polylysine-coated cover slips, allowed to settle for an additional 15 min, and then processed as above for viewing.

For investigating the ultrastructure of flagella, the virulent *Y. pseudotuberculosis* species, with *Y. pestis* serving as a control species, were grown in heart infusion broth at either 22 or 37°C for 48 h. The organisms were then fixed in 2% glutaraldehyde in culture medium, and single drops of the culture were transferred to polylysine-coated cover slips and processed in the same manner as described earlier for *Y. pestis*.

RESULTS AND DISCUSSION

Y. pestis. Virulent *Y. pestis* (strain T1) organisms grown at 22°C for different incubation periods yielded small quantities of fraction 1 envelope antigen, detectable as a particulate coating on the bacterial surfaces and the surrounding milieu. Figures 1, 2, 5, and 7 are scanning electron micrographs of a series incubated for 24, 24, 48, and 72 h, respectively, at 22°C. The morphological aspects of the microcolony (Fig. 1) revealed structures similar to the magnified single cells depicted in Fig. 2, 5, and 7. Here the bacilli are seen as sharply delineated rods with fairly smooth surfaces at 24- and 48-h incubation times (Fig. 2 and 5). A few particles coat the surfaces of cells incubated for 72 h, and some spill into the extracellular space (Fig. 7); but in comparison to the amount of antigen produced by cells incubated for only 24 h at the higher temperature, the yield of surface antigen is scant.

When the same virulent *Y. pestis* T1 was grown on heart infusion blood agar for 24 to 72 h at 37°C, the abundant granular particles covering the surfaces of bacterial cells could be clearly visualized (Fig. 3, 4, 6, 8, and 12). These surfaces seemed rough and lumpy, with particle fragments falling into the outer environment. The longer the incubation time, the more surface particles were present. That the scattered particles and aggregates, as well as particulate strands in the extracellular space, represented the soluble envelope antigen (Fig. 8 and 12), was verified by the appearance of identical particulate material, including the strands (arrows), in a micrograph of the 1% soluble fraction 1 envelope antigen preparation (Fig. 11). Moreover, this observation is further supported by the contention of previous plague researchers (4, 10, 15) that more envelope antigen can be recovered from cells and supernatant fluid from cultures grown at 37°C for 72 h. The considerable prominence of the particles, both on the cell surfaces and outside, is worth comment since the antigen is essential for preparing immunogenic cultures of *Yersiniae* for immunization against plague.

The envelope antigen is normally soluble and can be washed from the cell surfaces with distilled water (9). However, by fixing the cultures in situ before washing, we believe that we preserved more of the surface antigen. The fragments observed around the organisms were mostly aggregates of antigen particles, which either had first dissolved and then become reprecipitated during processing, or had been knocked from the surfaces of cells.

Habig et al. (11), by means of acrylamide gel electrophoresis, demonstrated that fraction 1 antigen consists of a heterogenous protein population and suggested that *Y. pestis* fraction 1 contains a series of molecular aggregates with identical subunits. In this connection, Bennett and Tornabene (3) recently reported that the soluble fraction 1 antigen of *Y. pestis* exists as aggregates of molecular weights of more than 300,000, and each aggregate can be further dissociated into a single antigenic subunit with a molecular weight of 15,000 to 17,000 by a "dissociation" treatment with 0.1% mercaptoethanol in 0.25% sodium dodecyl sulfate at 95°C for 5 min. Hence, our SEM studies would appear to have visually confirmed these physicochemical observations (see Fig. 11 and 12).

It should be noted that those bacilli that produced only slight surface antigen were surrounded by few (if any) free particles (Fig. 7). The strings (arrows) brokenly encircling the organisms in Fig. 8 and 12 (and also in Fig. 11, a micrograph of highly purified fraction 1 solution) were also mostly made up of the antigen, if one examines them carefully with a low-magnification lens. Figure 6 (a 48-h culture) exposes as much granular surface component as cultures grown for 72 h, which was anticipated. But the free particles and aggregates were seen much less often than in others (e.g., 72-h cultures at 37°C).

Y. pseudotuberculosis. Occasionally, in the study of *Y. pseudotuberculosis* and *Y. pestis*, particular attention was focused on differentiating between the two. Obviously, this is a

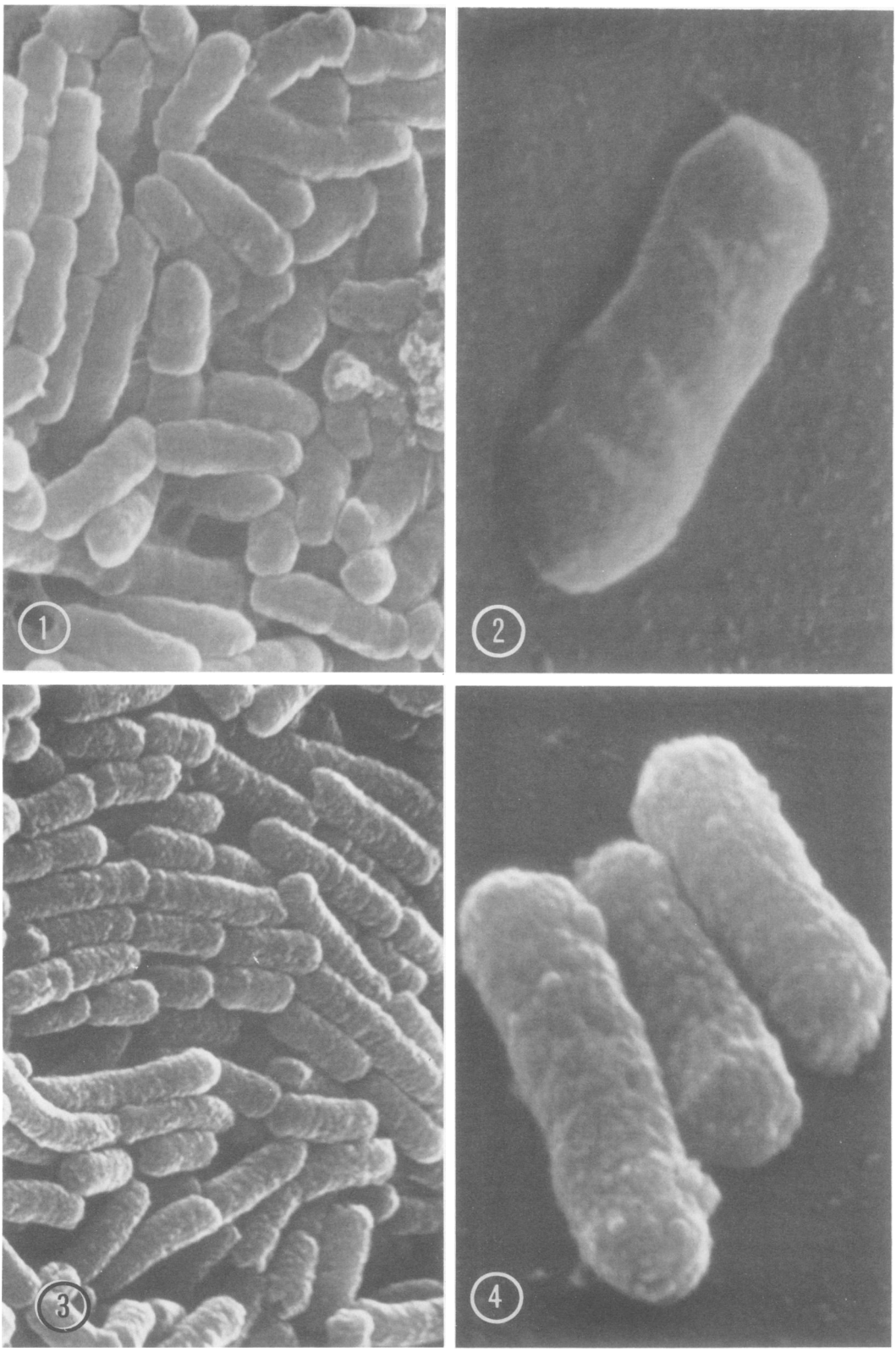
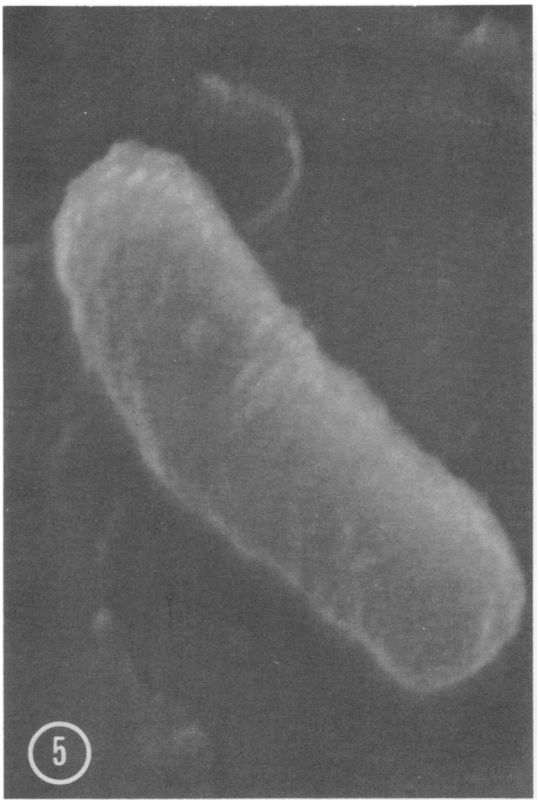
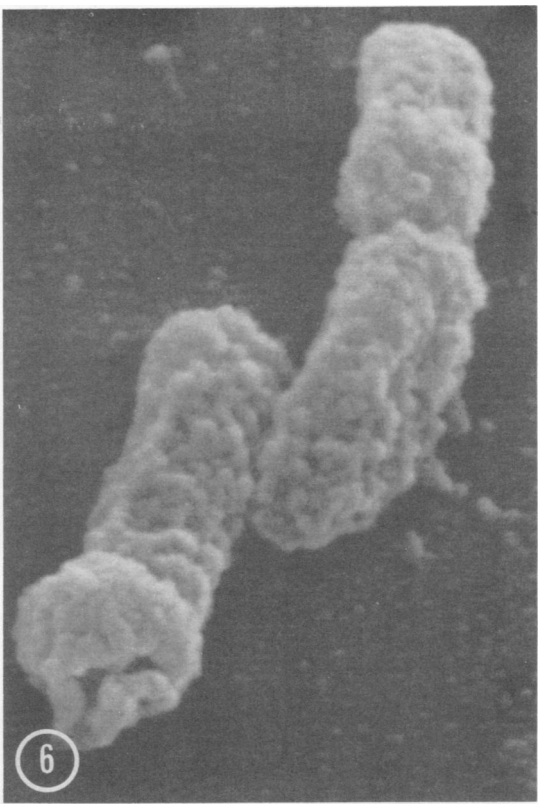
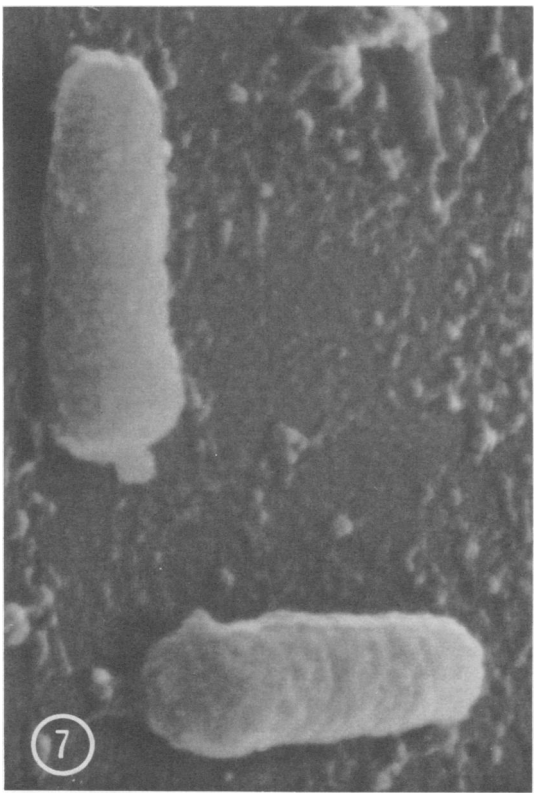


FIG. 1. *Scanning electron micrograph of a Y. pestis T1 colony grown at 22°C for 24 h. ×20,000.*
FIG. 2. *A single Y. pestis bacillus grown at 22°C for 24 h. ×59,000.*
FIG. 3. *Y. pestis colony cultured at 37°C for 24 h. ×15,000.*
FIG. 4. *Y. pestis cultured at 37°C for 24 h. Note the "lumpy" surfaces of the organisms. ×43,000.*







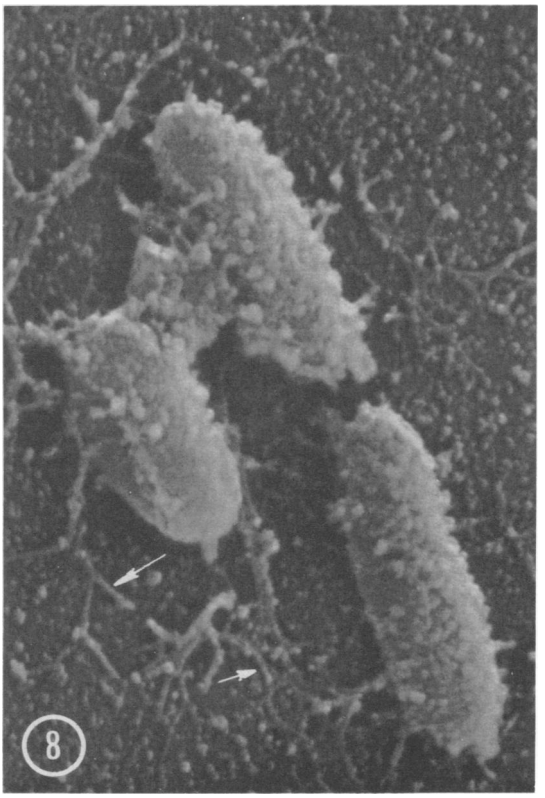
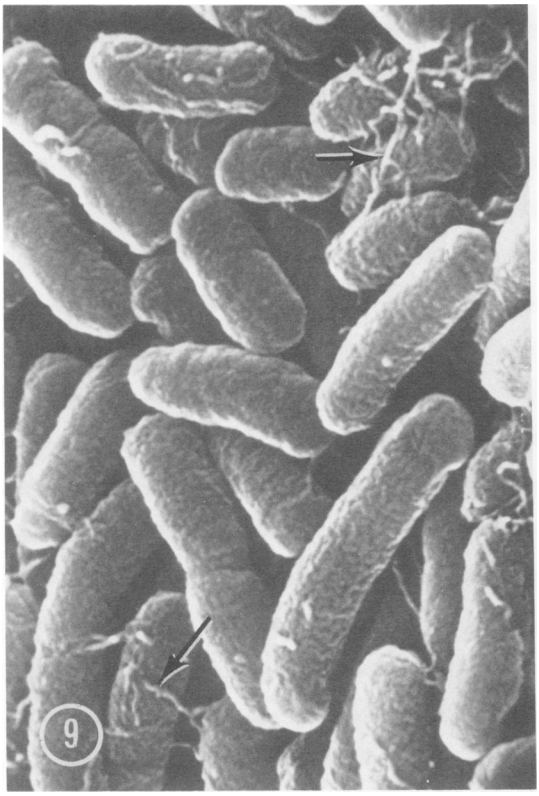


FIG. 5. *Y. pestis grown at 22°C for 48 h. ×62,000.*

FIG. 6. *Y. pestis grown at 37°C for 48 h. Compare the plentiful surface particles with the scanty amount in Fig. 5. ×40,000.*

FIG. 7. *Y. pestis grown at 22°C for 72 h. The particles on their surfaces and encircling the two bacilli represent the envelope antigen. ×55,000.*

FIG. 8. *Y. pestis grown at 37°C for 72 h. Observe the abundance of particulate antigen, both on the bacilli and in the substrate. Arrows indicate strings of antigen. ×30,000.*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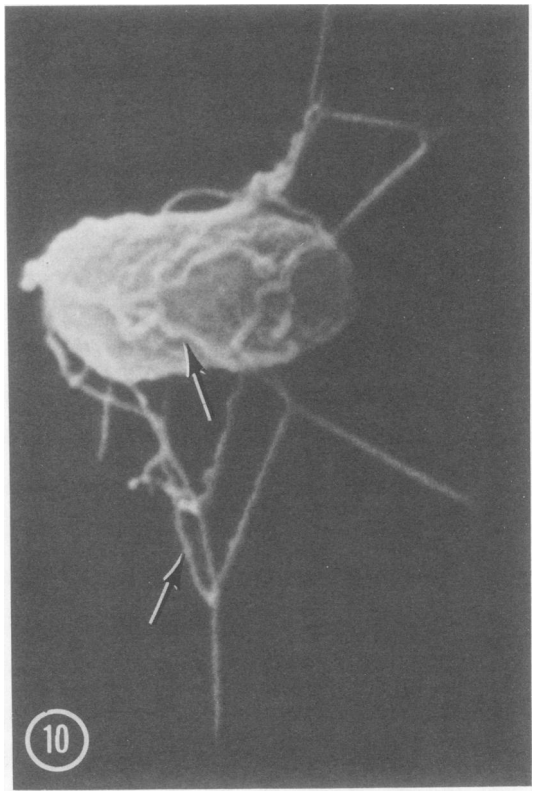
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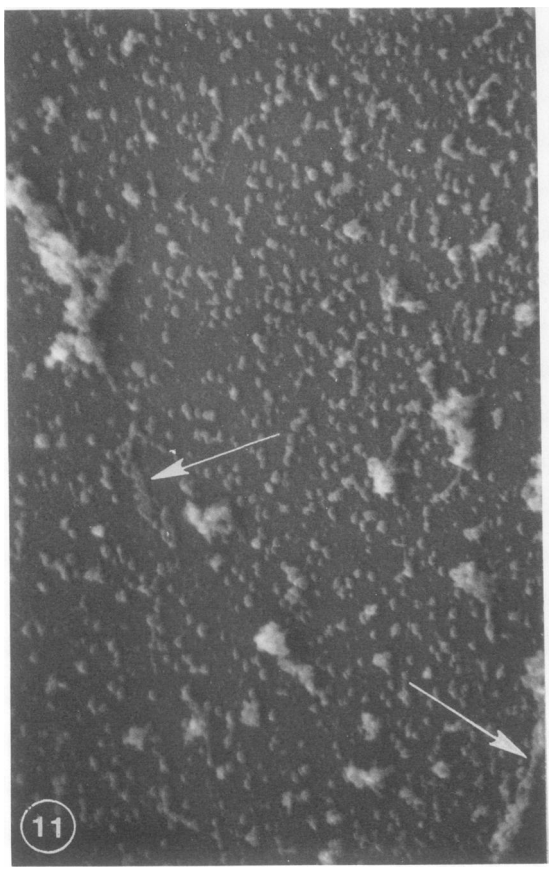
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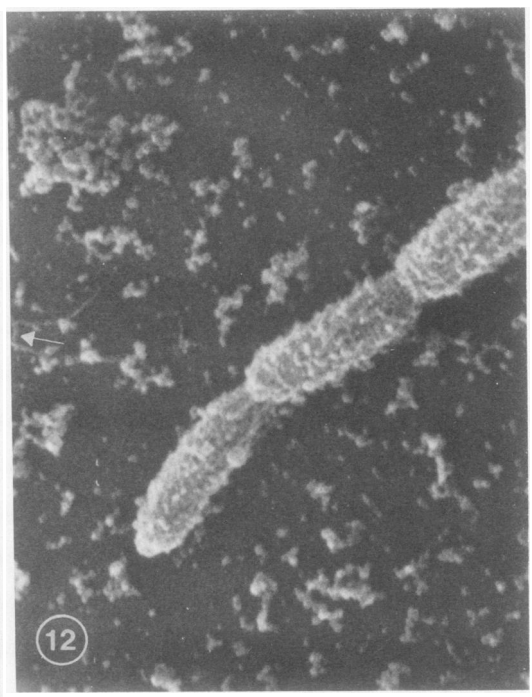
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matter of utmost importance when the investigator is working in an "quiescent" plague area or in a previously infected region (13). Frequently it is difficult to distinguish between these two gram-negative bacteria with the light microscope, and their intimate immunological relationship makes distinction by conventional serological tests difficult. Our differentiation of these two species by ultrastructural surface morphology is therefore significant—i.e., the presence of flagella in *Y. pseudotuberculosis* at 22 but not at 37°C or at any other temperature for growing plague bacilli (16) (micrographs not shown here), and the lack of these organelles in *Y. pestis* at all temperatures and incubation periods. In 1965, Quan et al. (13) demonstrated flagella on the cell surfaces of *Y. pseudotuberculosis* (reported, however, as *P. pestis*) by transmission electron microscopy, and then amended their designated plague bacillus to the *P. pseudotuberculosis* strain. However, this is an arduous and time-consuming method; SEM is both faster and more reliable. Our observation of flagella on the surfaces of *Y. pseudotuberculosis* organisms by SEM affords a new and quicker technique for distinguishing the two species, a method which in all likelihood has added another dimension to our morphological knowledge of plague and the pseudotuberculosis bacillus.

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LITERATURE CITED

1. **Amies, C. R.** 1951. The envelope substance of *Pasteurella pestis*. Br. J. Exp. Pathol. 32:259–273.
2. **Baker, E. E., H. Sommer, L. E. Foster, E. Meyer, and K. F. Meyer.** 1947. Antigenic structure of *Pasteurella pestis* and the isolation of a crystalline antigen. Proc. Soc. Exp. Biol. Med. 64:139–141.
3. **Bennet, L. G., and T. G. Tornabene.** 1974. Characterization of the antigenic subunits of the envelope protein of *Yersinia pestis*. J. Bacteriol. 117:48–55.
4. **Chen, T. H.** 1965. The antigenic structure of *Pasteurella pestis* and its relationship to virulence and immunity. Acta Trop. 22:97–117.
5. **Chen, T. H., S. S. Elberg, J. Boyles, and M. A. Velez.** 1975. *Yersinia pestis:* correlation of ultrastructure and immunological status. Infect. Immun. 11:1382–1390.
6. **Chen, T. H., and K. F. Meyer.** 1966. An evaluation of *Pasteurella pestis* Fraction-1-specific antibody for the confirmation of plague infections. Bull. W.H.O. 34:911–918.
7. **Chen, T. H., S. F. Quan, and K. F. Meyer.** 1952. Studies on immunization against plague. II. The complement fixation test. J. Immunol. 68:147–158.
8. **Cohen, A., D. Marlow, and G. Garner.** 1968. A rapid critical point method using fluorocarbons (Freons) as intermediate and transitional fluid. J. Microsc. (Oxford) 7:331–342.
9. **Crocker, T. T., T. H. Chen, and K. F. Meyer.** 1956. Electron microscopic study of the extracellular materials of *Pasteurella pestis*. J. Bacteriol. 72:851–857.
10. **Englesberg, E., and J. B. Levy.** 1954. Studies on immunization against plague. VI. Growth of *Pasteurella pestis* and the production of the envelope and other soluble antigens in a casein hydrolyzate mineral glucose medium. J. Bacteriol. 67:438–449.
11. **Habig, W., B. W. Hudson, J. D. Marshall, Jr., D. C. Cavanaugh, and J. H. Rust, Jr.** 1971. Evidence for molecular heterogeneity of the specific antigen (fraction-I) of *Pasteurella pestis*. Infect. Immun. 3:498–499.
12. **Meyer, K. F., J. A. Hightower, and F. R. McCrumb.** 1974. Plague immunization. VI. Vaccination with the Fraction I antigen of *Yersinia pestis*. J. Infect. Dis. Suppl. 129:S41–S45.
13. **Quan, S. F., W. Knapp, M. I. Goldenberg, B. W. Hudson, W. D. Lawton, T. H. Chen, and L. Kartman.** 1965. Isolation of a strain of *Pasteurella pseudotuberculosis* from Alaska identified as *Pasteurella pestis:* an immunofluorescent false positive. Am. J. Trop. Med. Hyg. 14:424–432.
14. **Rowland, S.** 1914. The morphology of the plague bacillus. J. Hyg. (Camb.) 13 (Plague Suppl. III):418–422.
15. **Schütze, H.** 1932. Study in *B. pestis*. I. Antigen and immunity reactions of *B. pestis*. Br. J. Exp. Pathol. 13:284–288.
16. **Wilson, G. S., and A. A. Miles (ed.).** 1975. Topley and Wilson's principles of bacteriology & immunity, 6th ed. The Williams & Wilkins Co., Baltimore.

FIG. 9. *Colony of Y. pseudotuberculosis grown at 22°C for 48 h. Arrows designate flagella. ×24,000.*

FIG. 10. *Y. pseudotuberculosis bacillus cultured at 22°C for 48 h. Note the prominent flagella (arrows). ×43,000.*

FIG. 11. *Highly purified fraction 1 envelope antigen of Y. pestis. Particles and aggregates are scattered throughout the field of vision. Arrows indicate antigen strands. ×15,000.*

FIG. 12. *Y. pestis grown at 37°C for 72 h. Numerous antigen particles, as well as some aggregates, are apparent both on the bacilli and in the substrate. The arrow points out an antigen string. ×10,000.*